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The overall goal of this project is to develop an understanding of tRNA gene structure and transcript processing in the halophilic Archaebacteria. During the second year of these studies we have extended our analysis of the processing of the halobacterial intron containing $tRNA^{Trp}$ precursor. Intron endonuclease has been found to have unique substrate recognition properties and progress has been made in developing a tRNA ligase assay using the products of this reaction. Intron endonuclease activity has also been detected in other Archaebacteria. Continued purification of RNaseP, the enzyme which removes 5' flanking sequences from tRNA primary transcripts, indicates that this enzyme may have a RNA component. Searches for stable intron containing precursor tRNAs in the halophilic Archaebacteria suggest that $tRNA^{Trp}$ may be the only interrupted tRNA gene in these organisms.

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PRINCIPAL INVESTIGATOR: Dr. Charles J. Daniels

CONTRACTOR: The Ohio State University, Columbus, Ohio

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START DATE: 1 August 1986

RESEARCH OBJECTIVE: To determine the mechanism of tRNA intron processing in the halophilic archaebacteria; characterize the enzyme responsible for the removal of 5'-flanking sequences from tRNA gene primary transcripts; examine the structure and distribution of tRNA introns in the halophilic archaebacteria.

PROGRESS:

A. Intron Processing. We have chosen the *H. volcanii* and closely related *H. mediterranei* intron containing $tRNA^{Trp}$ precursors as model substrates to investigate intron processing the Archaebacteria. Previously we had established an *in vitro* assay for the tRNA intron endonuclease using transcripts of these genes produced from T7 RNA polymerase transcription vectors. The enzyme was partially purified and shown to be capable of precise and accurate cleavage of the *in vitro* produced precursors. During this past year, we have extended our studies on the mechanism of intron processing. Further characterization of the endonuclease reaction products has indicated that phosphodiester bond cleavage results in the formation of 2',3' cyclic phosphate and 5' hydroxyl termini, similar to the eukaryotic tRNA intron endonuclease. In spite of this similarity, substrate recognition by the halophilic endonuclease was found to be distinctly different from its eukaryotic counterpart. In eukaryotes, recognition of intron containing tRNA precursors requires the presence of intact mature tRNA structure. The enzyme

interacts specifically with the anticodon stem, D stem and extra arm positions of the precursor and may use a measurement mechanism, from the central portion of the molecule to identify cleavage sites. We have prepared a number of deleted substrates lacking either exon or intron sequences and examined their activities as substrates (Fig. 1). These molecules either lacked regions of the exons preventing the formation of mature tRNA structures (Δ13, Δ1311, Δ13115), portions of the intron (Δ167) or both (Δ1311-10, Δ1311-24). From these studies we have concluded that only those exon sequences near the cleavage site are required (anticodon stem and loop); the presence of mature tRNA-like structure, a requirement for recognition by the eukaryotic endonuclease, is not required. The large intron, while not absolutely required, does affect the accuracy and efficiency of the cleavage reaction.

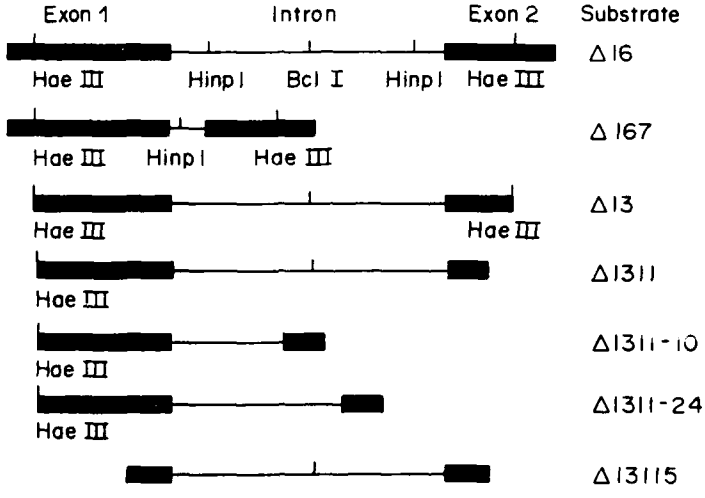

Figure 1. *H. volcanii* $tRNA^{Trp}$ intron endonuclease active substrates. The parental substrate Δ16 was derived from the *H. mediterranei* $tRNA^{Trp}$ gene. Removal of the intron specific HinpI fragment gave the substrate Δ167. The *H. volcanii* $tRNA^{Trp}$ gene was the source of the partial exon deleted substrate Δ13. Additional exon sequences were removed from Δ13 by exonuclease III deletion or site specific mutagenesis to yield the substrates Δ1311 and Δ13115. Partial deletions of intron sequences by Bal31 digestion from the BclI site of Δ1311 gave the substrates Δ1311-10 and -24. Exon sequences are indicated by boxes and intron by the solid lines.

Since the halobacterial endonuclease would not cleave a typical eukaryotic intron containing pretRNA, $pretRNA^{Phe}$, this enzyme must require a specific sequence or structure. Among potential structural interaction possible in the deleted substrates, those with extended anticodon helices were most energetically favorable. These structures were reminiscent of the RNaseIII cleavage sites found in rRNA operon primary transcripts. An

examination of RNaseIII cleavage sites in the halobacteria indicated sequence and structural similarity between $tRNA^{Trp}$ intron cleavage and the RNaseIII processing site of 23S rRNA (Fig. 2). To determine whether the endonuclease would recognize this RNA, a model substrate mimicking the halobacterial 23S rRNA RNaseIII cleavage site was prepared (Fig. 3). When assayed under standard conditions, the intron endonuclease was capable of cleaving this molecule. This suggested that the extended helix structure in which the cleavage sites are localized, the sequence 5'-UCCAG-3', or both are important for recognition.

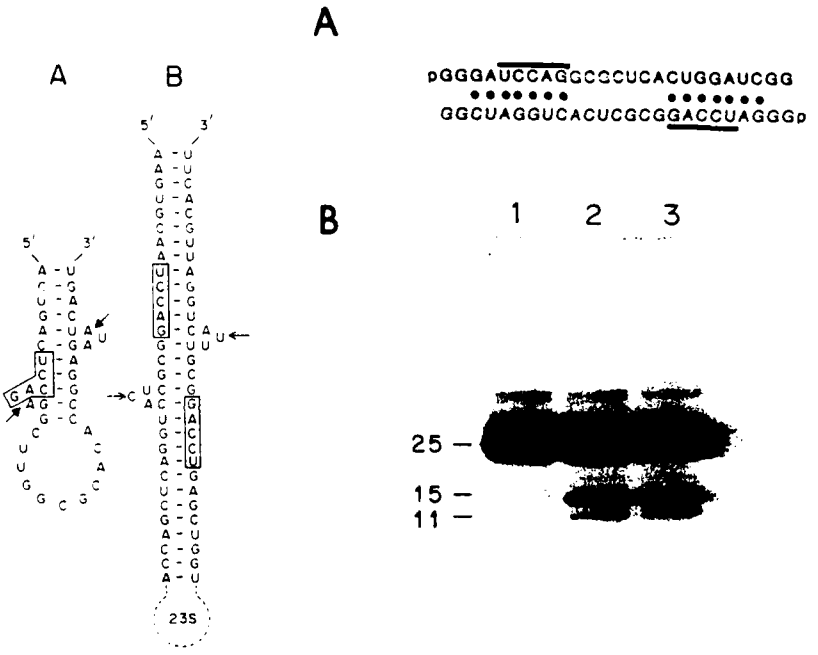

Figure 2. Comparison of cleavage sites for the $tRNA^{Trp}$ intron endonuclease and 23S rRNA RNaseIII. A. The exon intron boundaries of the Δ167 substrate. B. The proposed RNaseIII cleavage site of the *H. cutirubrum* 23S rRNA. Boxed nucleotides are conserved between these RNAs and include the $tRNA^{Trp}$ anticodon sequence CCA. Cleavage sites for intron endonuclease and RNaseIII are indicated by solid and open arrows, respectively.

Figure 3. Cleavage of a RNaseIII model substrate. A. Double stranded structure of the self complimentary RNA representing the 23S rRNA RNaseIII cleavage site of *H. cutirubrum*. RNA was obtained by T7 RNA polymerase directed *in vitro* transcription. B. Cleavage of the model substrate labeled with $\alpha[^{32}P]ATP$ with partially purified *H. volcanii* $tRNA^{Trp}$ intron endonuclease. Lane 1, uncut; Lane 2, cleavage at 25°C; and Lane 3 cleavage at 37°C.

Utilizing the products of the $tRNA^{Trp}$ endonuclease reaction, we have begun studies on the ligation of tRNA half molecules, the second step in the processing pathway. Products similar in size to those predicted for combined endonuclease and ligase reactions can be detected in freshly prepared crude

extracts (Fig. 4). However, this activity is rapidly inactivated. The lack of ligase activity is not unique to halobacteria. We have also been unable to detect significant amounts of ligase activity in extracts of *Thermoplasma*, *Methanosarcina* and *Sulfobolus*. Studies are currently underway to examine the structure of this product to determine whether correct ligation has occurred.

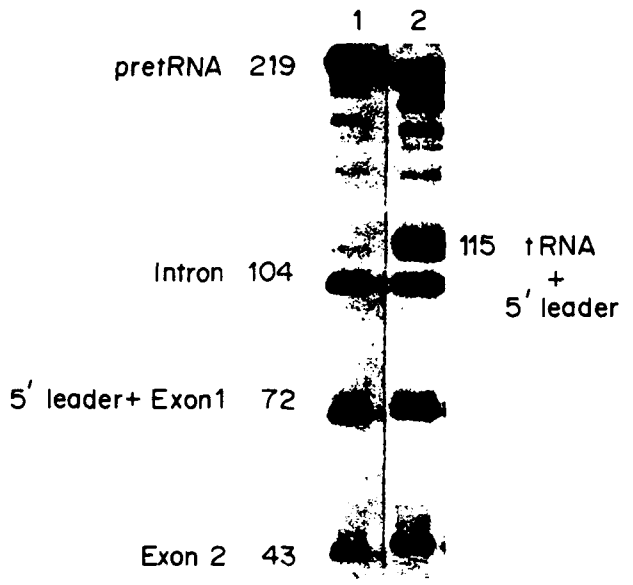

Figure 4. Combined endonuclease and ligase activities. Uniformly labeled Δ16 RNA was processed with the partially purified endonuclease preparation (Lane 1) or with a freshly prepared 10% PEG precipitate fraction of a *H. volcanii* S30 extract (Lane 2). The proposed ligation product (115 nt) is indicated.

B. Removal of 5' leader sequences by RNaseP. We have continued our characterization of the *H. volcanii* RNaseP enzyme. Using an *in vitro* generated substrate derived from the *H. volcanii* $tRNA^{Val}$ gene, we have established an assay for this enzyme and are developing strategies for the purification of this enzyme. While we have been unable to demonstrate a functional requirement for RNA by micrococcal nuclease treatment due to EGTA inhibition, we observe RNA species copurifying with the enzymatic activity. A large RNA of approximately 405 nucleotides and two smaller RNAs (140 and 105 nucleotides) are likely candidates for the RNaseP RNA (Fig. 5). The smaller RNAs have been shown by Southern analysis to be nonribosomal in origin and have been localized to a unique PstI restriction fragment. We have identified several proteins which copurify with these RNAs. Among these is a 17,000 daltor protein which is similar in size to the protein component of the eubacterial RNaseP complex.

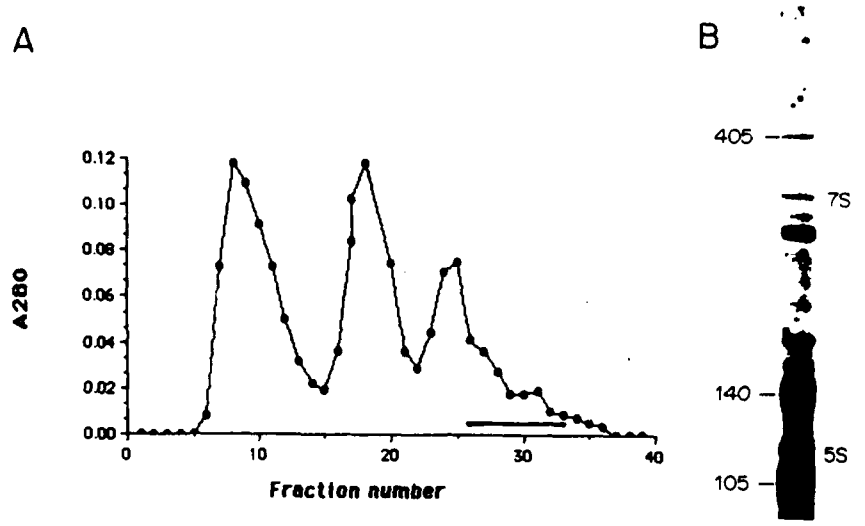

Figure 5. Fractionation of RNaseP activity by gel filtration. A. Gel filtration of a 6-10% PEG precipitate from a *H. volcanii* S30 extract on Sepharose 4B. Active fractions are indicated by the solid bar. B. pCp end labeling of RNAs from fraction 31. RNAs which copurify with RNaseP activity are indicated.

C. Identification of intron containing tRNA genes. Using Northern analysis, two approaches have been used to search for additional intron containing precursor tRNA molecules in the *H. volcanii*. The presence of intron containing pretRNA^{Trp} molecules in total RNA preparations suggested that other precursors may also be present in detectable amounts in these preparations. Complimentary tDNAs were prepared by first adding poly A tails to the total tRNA population followed by second strand synthesis using reverse transcriptase and an oligo dT primer. These ctDNAs hybridized to mature tRNAs and a species which was similar in size to the predicted intron containing pretRNA^{Trp} molecule. A second probe was also used. Based on the sequences of 44 *H. volcanii* tRNAs, a consensus antitRNA oligonucleotide probe was synthesized. Like the ctDNAs, this probe hybridized to mature tRNAs and intron containing pretRNA^{Trp} sized molecules. These data do not exclude the presence of other intron-containing pretRNAs, however they do suggest the possibility that tRNA^{Trp} may be the only interrupted tRNA gene in this organism. A comprehensive sequence analysis of tRNA genes in this organism appears to be the only method to resolve this question.

In an examination of other organisms for interrupted tRNA genes and processing activity, we have detected tRNA^{Trp} endonuclease activity in

extracts of *Thermoplasma* (Fig. 6A), suggesting that the $tRNA^{Trp}$ gene or possibly other tRNA genes in this organism are interrupted by an intron. This activity appeared to be specific for the Archaebacterial substrate. Like the halobacterial enzyme, it did not cleave the yeast $tRNA^{Phe}$. Using a halobacterial $tRNA^{Trp}$ exon probe, we have identified restriction fragments bearing these sequences (Fig. 6B). Sequences related to the halobacterial $tRNA^{Trp}$ intron did not hybridize, even under low stringency conditions, indicating that the $tRNA^{Trp}$ gene, if interrupted, had a different intron.

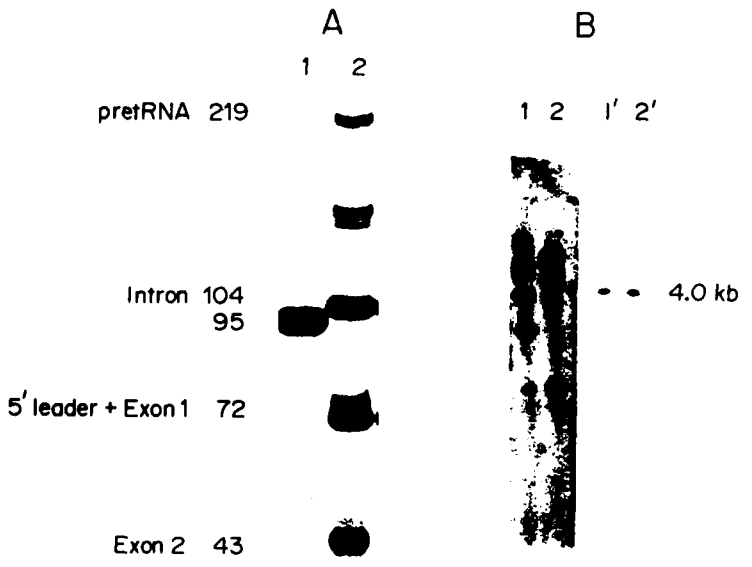

Figure 6. Endonuclease activity in *Thermoplasma*. A. Cleavage of uniformly labeled yeast $pretRNA^{Phe}$ (Lane 1) and *H. volcanii* $pretRNA^{Trp}$ Δ16 (Lane 2) by a crude lysate of *Thermoplasma*. B. Southern analysis of HindIII (Lane 1, 1') or PstI (Lane 2, 2') cleaved total *Thermoplasma* DNA probed with *H. volcanii* $tRNA^{Trp}$ sequences under high (Lanes 1 & 2) or low (Lanes 1' & 2') stringency conditions.

WORKPLAN:

A. Intron processing. In keeping with our goal to characterize the mechanism of tRNA processing, we will continue our efforts to develop a tRNA ligase assay. A possible factor which may influence the efficiency of the reaction may be a requirement for mature 5' and 3' termini. To examine this, we will use RNaseP to remove the 5' leader of $pretRNA^{Trp}$ and the yeast $pretRNA^{Phe}$ gene and yeast endonuclease to produce tRNA half molecules. These

latter molecules will have 5′ phosphate and 2′,3′ cyclic phosphate termini on the first exon and a 5′ hydroxyl and 3′ CCA residues on the second exon, features which are representative of the *in vivo* substrate and required by the yeast ligase *in vitro*. In an attempt to isolate stable activity, both high and low salt extracts of *H. volcanii* will be examined. Protease inhibitors and other stabilizing agents will also be employed.

We will continue our characterization of the substrate recognition properties of the endonuclease. Using site specific mutational analysis, we will determine whether this enzyme recognizes the $tRNA^{Trp}$ anticodon sequences, a specific structure or both. In parallel, we will use additional chromatographic procedures such as CM-cellulose, dye columns, poly I·C double stranded RNA columns and possibly affinity chromatography to further purify this activity. This is particularly important in view of the possibility that this enzyme may act on both tRNA and rRNA precursors.

B. RNaseP. Using Southern analysis and direct RNA sequencing, the relationship between the three RNAs which copurify with RNaseP activity will be investigated. These RNAs will then be used to identify and clone their corresponding genes. Comparative structural analysis of potential transcripts from these genes with other RNaseP molecules and reconstruction assays using *in vitro* produced RNA, in the presence and absence of added proteins, will be used to verify the role of this RNA in the reaction.

C. Occurrence of introns. Examination of tRNA intron distribution will focus on the $tRNA^{Trp}$ genes of *Thermoplasma*. This gene will be cloned using the *H. volcanii* gene probe. Sequence of this gene will indicate whether an intron is present and if the intron has structural or sequence similarity to the halobacterial gene. Additional tRNA genes will be cloned using end labeled *Thermoplasma* tRNA as probe and screened by Northern analysis for precursor forms. We will also continue in our efforts to clone tRNA genes from *H. volcanii* large (>90 kbp) BamHl tRNA-rich restriction fragments isolated by field inversion electrophoresis. These experiments remain part of the long term goal of this project, but must be considered lower in priority due to the uncertainty of finding additional introns.

PUBLICATIONS AND PRESENTATIONS:

1. Leo D. Thompson and Charles J. Daniels. A $tRNA^{Trp}$ Intron Endonuclease from *Halobacterium volcanii*: Unique Substrate Recognition Properties. J. Biol. Chem. Submitted.

2. James W. Brown, Charles J. Daniels and John N. Reeve. Gene Structure, Organization and Expression in Archaebacteria. Critical Reviews in Microbiology, CRC Press, in press.

This work has also been presented at three meetings:

3. Intron Processing in the Halophilic Archaebacteria. Leo D. Thompson and Charles J. Daniels. Canadian Institute for Advanced Research: Genome Evolution. September, 1987. Invited speaker.

4. The $tRNA^{Trp}$ Intron Endonuclease from *Halobacterium volcanii* Has Unique Substrate Recognition Properties. Leo D. Thompson and Charles J. Daniels. The Ohio State Biotechnology Conference. April, 1988.

5. Unique Substrate Recognition Properties of an Archaebacteria tRNA Intron Endonuclease. Leo D. Thompson and Charles J. Daniels. RNA Processing, Cold Spring Harbor Laboratory. May, 1988. Invited speaker.

TRAINING ACTIVITIES:

Two graduate students, Leo D. Thompson and Daniel T. Nieuwlandt and an undergraduate student, Jorge L. Acevedo, were supported by this grant. Mr. Acevedo is a minority student from Puerto Rico.

AWARDS:

Dr. C.J. Daniels was appointed as an Associate of the Canadian Institute for Advanced Research, April, 1988.